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High-density peptide microarray exploration of the antibody response in a rabbit immunized with a neurotoxic venom fraction

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Abstract

Polyvalent snakebite antivenoms derive their therapeutic success from the ability of their antibodies to neutralize venom toxins across multiple snake species. This ability results from a production process involving immunization of large mammals with a broad suite of toxins present in venoms. As a result of immunization with this wide range of toxins, many polyvalent antivenoms have a high degree of cross-reactivity to similar toxins in other snake venoms – a cross-reactivity which cannot easily be deconvoluted. As a proof of concept, we aimed at exploring the opposite scenario by performing a high-throughput evaluation of the extent of cross-reactivity of a polyclonal mixture of antibodies that was raised against only a single snake venom fraction. For this purpose, a venom fraction containing short neurotoxin 1 (SN-1; Uniprot accession number P01416, three-finger toxin (3FTx) family), which is the medically most important toxin from the notorious black mamba (*Dendroaspis polylepis*), was employed. Following immunization of a rabbit, a specific polyclonal antibody response was confirmed by ELISA and immunodiffusion. Subsequently, these

antibodies were investigated by high-density peptide microarray to reveal linear elements of recognized epitopes across 742 3FTxs and 10 dendrotoxins. This exploratory study demonstrates in a single immunized animal that cross-reactivity between toxins of high similarity may be difficult to obtain when immunizing with a single 3FTx containing venom fraction. Additionally, this study explored the influence of employing different lengths of peptides in high-density peptide microarray experiments for identification of toxin epitopes. Using 8-mer, 12-mer, and 15-mer peptides, a single linear epitope element was identified in SN-1 with high precision.

Keywords: Epitope mapping, single toxin immunization, three-finger toxin, short neurotoxin, *Dendroaspis polylepis*

Introduction

Each year worldwide, snakebite envenoming causes mortality and morbidity to an overwhelming number of victims, particularly in rural tropical parts of the world

(Gutiérrez et al., 2010; Harrison et al., 2011; Harrison and Gutiérrez, 2016). Despite recent biotechnological and medicinal chemistry developments within snakebite envenoming therapy, antivenom derived from the serum of immunized animals is still the only effective treatment option used clinically (Gutiérrez et al., 2011; Laustsen et al., 2016). Consisting of antibodies generated by the animal immune system, antivenoms are capable of neutralizing the toxic effects of the venoms used in the immunization procedure. In many cases, however, antivenom can also cross-neutralize venom(s) from other species than those included in the immunization mixture (Williams et al., 2011). This desirable quality, referred to as cross-reactivity or para-specificity, must be investigated for each antivenom in a preclinical setting and cannot necessarily be assumed *a priori*. Traditionally, preclinical efficacy and cross-reactivity have been studied using ELISA-based methods and *in vivo* models. More recently, the antivenomics protocol based on immuno-affinity chromatography has successfully been introduced as a tool to assess cross-reactivity and to predict efficacy of antivenoms prior to rodent testing (Pla et al., 2012; Gutiérrez et al., 2013, 2017). Although the antivenomics method has proven useful in describing antivenom cross-

reactivity on the protein family level, it lacks the capacity to reveal details on the amino acid residue level and to explain, in molecular detail, why some similar toxins are recognized equally well, while others are not. Due to this missing information, rational improvement of antivenoms can only be achieved through trial and error.

Aiming at obtaining further in-depth understanding of cross-reactivity on an amino acid residue-level, mapping of antibody binding sites (epitopes) can be performed. Ideally, such studies should entail structural elucidation of antibody antigen complexes (Van Regenmortel, 2009). However, less extensive identification of linear elements of epitopes has previously been found to be helpful in understanding the interaction(s) between antibodies and single snake toxins (Ferreira et al., 2006; Lomonte, 2012; De-Simone et al., 2013; Castro et al., 2015; Ramos et al., 2016; Schneider et al., 2016). Recently, we introduced high-density peptide microarray technology for high-throughput epitope mapping of toxins into the field of antivenom research, making it possible to study antivenom cross-reactivity on a larger scale (Engmark et al., 2017, 2016). So far, almost all epitope mapping studies of snake toxins have studied complex mixtures of antibodies, derived from pooling of sera of multiple animals immunized

with mixtures of several whole venoms. In studies of such complex systems, it is challenging to understand how the immune systems of the individual animals recognize single venom components and to elucidate how cross-reactivity is derived. One unanswered question remains regarding whether a polyvalent antivenom derives its broad cross-reactivity mainly from the injection of venom mixtures in each animal in the immunization process or from the pooling of sera from different immunized animals. Also, it is unclear to which extent the non-toxic venom components in the immunization mixture affect the immune response against the critical toxins. Hypothetically, a medically important toxin could go unnoticed by the animal immune system if the toxin has low immunogenicity, whereas less important, but immunogenic, venom components may give rise to a strong antibody response (Laustsen et al., 2017). Therefore, both the "polyvalent" recognition (based on pooled sera from animals individually immunized with complex mixtures of toxins) and the recognition originating from single animals against single toxins may provide important complementary information to understanding the complex phenomena underlying the production and performance of cross-reactive antivenoms. Potentially, such knowledge

of Nature's choice of linear epitope elements can also be exploited in the development of antivenoms by more targeted approaches. As an example, such approaches could involve the use of mixtures of monoclonal antibodies, where each antibody must be carefully engineered and selected to ensure desired cross-reactivity of the entire antivenom antibody cocktail. Investigation of immune responses of both pooled and single sera may therefore unlock new knowledge and create a better foundation for understanding the immunological interaction between venom and antivenom.

Aiming at exploiting the potential of the high-throughput microarray platform in characterizing the cross-reactivity of a polyclonal antibody response in a single animal towards a single toxin, a rabbit was immunized with a venom fraction predominantly containing the medically most important toxin from the notorious black mamba (*Dendroaspis polylepis*), short neurotoxin 1 (SN-1; Uniprot accession number P01416) (Laustsen et al., 2015b; Petras et al., 2016). Specific recognition of SN-1 by the polyclonal rabbit antibodies was confirmed by ELISA and immunodiffusion. Subsequently, this response was investigated by high-density peptide microarray to reveal linear epitope elements and cross-reactivity to other three-finger toxins (3FTxs)

to evaluate the extent of cross-recognition obtained in the immunization process. Furthermore, the high-density peptide microarray setup employed here allowed investigation of the influence of the length of peptides on a microarray on the signal output.

This study is the first to explore the immune response from a single animal immunized with a single snake venom toxin by high-density peptide microarray technology and therefore exploratory in nature and limited in its scope and conclusions.

Methods

Preparation of rabbit anti-SN-1 antiserum

Dendroaspis polylepis venom was obtained from Latoxan SAS, Valence, France. The venom is a pool obtained from several specimens collected in Kenya. Fractions of short neurotoxin 1 (SN-1) were isolated as described (Laustsen et al., 2015b) and pooled. Purity of SN-1 was determined using MALDI-TOF. In brief, the sample was mixed with an equal volume of a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile/water containing 0.1% trifluoroacetic acid and 1 µL was spotted onto an Opti-TOF plate, and analyzed by MALDI-TOF on an Applied Biosystems 4800 Plus instrument in positive linear mode. Data acquisition was performed by accumulation of 500 laser shots with a laser intensity of 3800. The peaks were integrated and the relative purity of SN-1 was estimated to 85 mol%, on the basis of ion peaks intensity.

A white New Zealand rabbit (approx. 2.5 kg) was immunized by injection of SN-1 emulsified in Freund's adjuvant, or adsorbed to aluminum hydroxide, according to the scheme in Table 1, following protocols approved by the Institutional Committee for the

Use and Care of Animals (CICUA), University of Costa Rica. The immunization scheme was designed to mimic the immunization protocols used for antivenom production (Gutiérrez et al., 2005). The use of Freund's complete adjuvant was limited to one injection, followed by one injection of Freund's incomplete adjuvant, aiming to minimize the adverse effects of the adjuvant in the animal. The rabbit was bled 14 days after the last immunization. Serum was separated by centrifugation and stored at –20 °C.

Table 1: Immunization protocol for the rabbit employed in this study.

Day	*Dose SN-1 (µg)*	*Adjuvant*	*Route*
0	20	Freund's complete	s.c.
15	30	Freund's incomplete	i.m.
27	60	$Al(OH)_3$	s.c.
47	120	$Al(OH)_3$	i.m.
67	120	$Al(OH)_3$	s.c.
90	60	$Al(OH)_3$	i.m.
106	120	$Al(OH)_3$	i.m.

144 **ELISA protocols**

145 The two ELISA experiments with either venom fractions or whole venoms were
146 performed in two different labs containing different equipment, why the two assays
147 were performed following two slightly different protocols.

148 For investigating the response of anti-SN-1 antiserum against venom fractions,
149 the wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with
150 0.6 µg of each HPLC venom fraction dissolved in 100 µL PBS. Wells were blocked by
151 adding 100 µL of PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) and
152 mixed at room temperature for 1 h. Plates were washed five times with PBS. 100 µL of
153 a 1:2000 dilution of anti-SN-1 serum in PBS containing 2% BSA was added to each well
154 in triplicates, and incubated for 2 h, followed by five additional washings with PBS.
155 Then, 100 µL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-
156 horse IgG (whole molecule)-alkaline phosphatase in PBS containing 2% BSA) was
157 added to each well. The plates were incubated for 2 h, and then washed five times
158 with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM ZnCl2, 1 mM MgCl2, pH 7.4). Color
159 development was achieved by the addition of 100 µL p-nitrophenyl phosphate (1

160 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8). The absorbances at 405 nm were
161 recorded (Multiskan FC, Thermo Scientific) at several time intervals.

162 For investigation of anti-SN-1 antiserum cross-reactivity to whole venoms, wells in
163 MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 1 µg of whole
164 venom dissolved in 100 µL PBS. Wells were blocked by adding 100 µL PBS containing
165 2% (w:v) bovine serum albumin (BSA, Sigma) and mixed at room temperature for 1 h.
166 Plates were washed five times with PBS. Lyophilized rabbit sera (pre-immune or anti-
167 SN-1) were reconstituted to a concentration of 86 µg/ml in PBS containing 2% BSA, to
168 reach a similar antibody concentration as in the other ELISA, and 100 µL was added to
169 the corresponding well, and incubated for 1 h, followed by five additional washings
170 with PBS. Then, 100 µL of a 1:2000 dilution of anti-rabbit IgG (whole molecule)-
171 peroxidase (Sigma A8275; produced in goat) in PBS + 2% BSA was added to each well.
172 The plates were incubated for 1 h, and then washed five times with PBS. Color
173 development was achieved by addition of 100 µL OPD solution (10 mg *o*-
174 phenylenediamine dihydrochloride in 12 mL distilled water containing 20 µL of 30%

175 H_2O_2). After 4 minutes of color development, the reactions were stopped by addition
176 of 100 µL 0.5 M H_2SO_4, and the absorbances at 490 nm were recorded.
177

178 **Double immunodiffusion of anti-SN-1 serum against SN-1 and whole venoms**

179 Agarose was dissolved in 30 mL of PBS, to attain 1% concentration, and poured into a
180 Petri dish. Six holes were punched in the gel, and 50 µL of anti-SN-1 serum was placed
181 in the center well, while 30 µL of PBS containing SN-1 (0.5 mg/mL) or whole venom
182 from a snake (2 mg/mL) was added to each of the surrounding wells. After overnight
183 incubation at room temperature, the agarose gel was photographed using a
184 ChemiDoc® recorder and ImageLab® software.
185

186 **Design of high-density peptide microarray**

187 Based on the sequences of 740 3TFxs and 10 dendrotoxins found in the Uniprot
188 database (Boutet et al., 2016) at the time of the microarray design (20th May 2015), an
189 *in silico* library of 8-mer, 12-mer and 15-mer peptides was generated. The peptides

were tiled over the protein sequences with an offset of just one residue resulting in 151,884 peptides. The library was curated for redundant (non-unique) peptides, leaving 64,612 unique peptides for synthesis on the peptide microarray in triplicates. The individual peptides in the library were assigned random positions on the microarray to minimize local intensity biases. A list of the Uniprot accession numbers, a fasta file, and list of peptides (including results) are shared under the CC BY 4.0 license https://doi.org/10.6084/m9.figshare.5324914.v1.

Peptide microarray hybridization

The peptide microarray was produced by Schafer-N (Copenhagen, Denmark) using mask-less photolithographic synthesis adapted to solid-phase peptide synthesis with the C-terminal residue linked to the surface of the array, as previously described (Buus et al., 2012). The microarray was first incubated for 1.5 hours with 50 µg/mL pre-immune rabbit serum in 0.05 M Tris/acetate (Trizma® base, Sigma-Aldrich), pH 8.0, 0.1% v/v Tween 20, 1 g/L Bovine serum albumin (dilution and washing buffer). After washing, the array was incubated with 1 µg/mL of goat anti-rabbit IgG (H+L)

206 conjugated with Cy3 (Jackson ImmunoResearch) at room temperature for 1.5 hours.
207 Washing procedure was repeated to remove unbound antibody conjugate, and an
208 image was recorded at the wavelength 570 nm using an InnoScan900 microarray
209 scanner (Innopsys) with an excitation wavelength of 550 nm. The microarray was then
210 washed and incubated with 50 µg/mL anti-SN-1 serum for 1.5 hours, followed by
211 further washing and a second incubation with the same antibody conjugate as above,
212 washed and recorded. Fluorescence intensity for each peptide field was calculated from
213 the resulting image files using proprietary software by Schafer-N.
214

215 **Analysis of microarray results**

216 To remove the influence of single outliers, the median absorbance signal of each
217 peptide was determined for each of the two rounds of incubation with rabbit serum
218 and fluorophore-labeled anti-rabbit IgG antibodies. Anti-rabbit IgG antibodies bound
219 after incubation with pre-immune rabbit serum might be able to catch additional
220 rabbit IgG not specific for the peptides synthesized in a peptide field (Carmona et al.,

2015). This "reincubation effect" for signals from unspecific binding was determined by correlation of the signals for all peptides.

The median signal obtained after incubation with anti-SN-1 serum was corrected with the median signal obtained after pre-immune serum incubation multiplied by the reincubation effect. The resulting score of each peptide were mapped to each toxin by the position of the peptide N-terminal residue in the toxin sequences. The results for the protein subfamily short-chain (type 1) α-neurotoxin of the 3FTxs were aligned using Clustal Omega (Sievers et al., 2014) and visualized as aligned binding profiles, as previously described (Engmark et al., 2017, 2016).

For further visualization of the results for the toxins best recognized in the study, each residue was assigned a new score corresponding to the sum of the three highest scores of an 8-mer, a 12-mer, and a 15-mer containing the amino acid residue. The results for SN-1 were mapped to the average NMR structure (Labhardt et al., 1988) using the R package Ppdb (Id'e, 2014) and the PyMOL molecular graphics system.

Results and discussion

ELISA-based cross-reactivity of anti-SN1 antiserum

Aiming at investigating the cross-reactivity profile of polyclonal antibodies resulting from the immunization of a single animal with a venom fraction primarily containing a single toxin, a white New Zealand rabbit was immunized with HPLC purified SN-1 (fraction 4 in Fig 1A). The purity of SN-1 in the HPLC venom fraction was determined to 85 mol% with trace amounts of two other small neurotoxins (Fig S1). Based on ELISA, the anti-SN-1 serum obtained from this rabbit could recognize the SN-1 as well as several other HPLC purified fractions of black mamba venom containing 3FTxs (Fig 1B). Furthermore, the antiserum was found to bind one or more components in the venom of the related eastern green mamba (*D. angusticeps*), while barely detectable (yet significant) signals were observed for the more distantly related cobra (*Naja* spp.) venoms and for the Central American coral snake (*Micrurus nigrocinctus*) venom (Fig 1C). Strong recognition of SN-1 was additionally confirmed for the anti-SN-1 serum by immunodiffusion, although recognition of whole venoms from both *D. polylepis* and

other snakes were not detected (Fig1D). The lack of recognition of other snake venoms devoid of SN-1 serves as controls, whereas the lack of visible recognition of *D. polylepis* whole venom is due to the low sensitivity of the immunodiffusion technique combined with the low abundance of SN-1 in *D. polylepis* venom (3.7%) (Laustsen et al., 2015b). This demonstrates that the antiserum is selective towards the SN-1 enriched venom fraction.

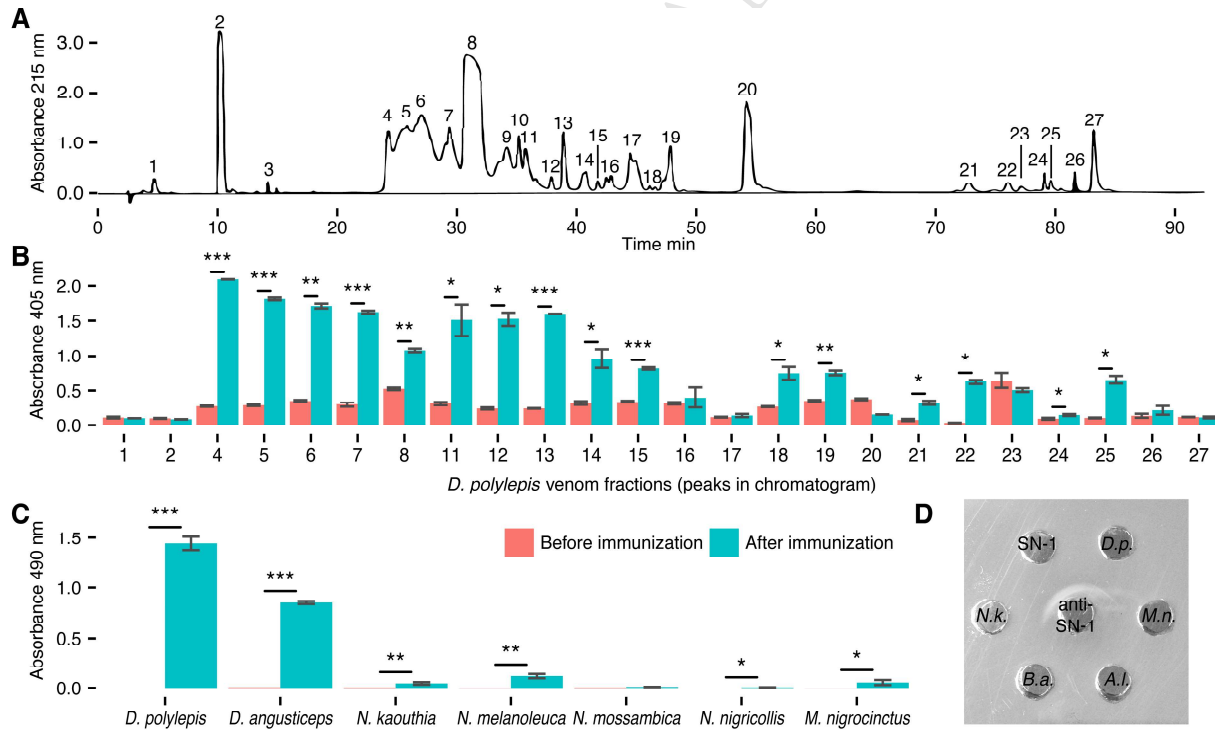

Figure 1. *HPLC purification of SN-1 and ELISA-based immunoprofiling of anti-SN1 antiserum.* ***A)*** *HPLC chromatogram of D. polylepis venom. Numbering of fractions follows the scheme described*

in (Laustsen et al., 2015b). SN-1 was found to be the main component in fraction 4. ***B)*** *Response against fractions isolated from D. polylepis venom.* ***C)*** *Response against whole venoms from D. polylepis and related elapid species. Asterisks denote p-values for one-sided Welch Two Sample t-test (three asterisks: $P \leq 0.001$, two asterisks: $0.001 < P \leq 0.01$, one asterisk: $0.01 < P \leq 0.05$).* ***D)*** *Double immunodiffusion of anti-SN-1 serum against SN-1 (concentration: 0.5 mg/mL) and whole venoms (concentrations: 2 mg/mL): D. polylepis (D.p.), N. kaouthia (N.k.), Micrurus nigrocinctus (M.n.), Bothrops asper (B.a.), and Aipysurus laevis (A.l.).*

Peptide microarray experiments reveal high selectivity for short neurotoxin 1

Sparked by the observed cross-reactivity in ELISA and double immunodiffusion experiments (Fig 1B-C), the rabbit antiserum was investigated in a high-density peptide microarray experiment containing peptides corresponding to all available sequences from 3FTxs in the Uniprot database (Boutet et al., 2016). The aim was to elucidate how broad the polyclonal antibody response of this single animal was as a case investigation of the extent of cross-reactivity derived from immunization with a single venom fraction. Importantly, as epitopes may not contain linear elements long enough

277 to be detected in a peptide array setup, the following discussion is limited to what can
278 be measured in the setup and the results are likely not to reveal a complete picture of
279 the interactions between toxin and polyclonal antibodies.

280 To increase sensitivity of the peptide microarray study, long (15-mer) peptides
281 was included in the setup as long peptides are more flexible than shorter peptides and
282 might more easily be induced by antibodies to fold into conformations corresponding
283 to the antigen surface. On the other hand, the inclusion of shorter (8-mer and 12-mer)
284 peptides on the microarray may increase precision of localization of highly recognized
285 linear epitope elements and may allow identification of two individual linear element
286 co-localized on a 15-mer peptide (Buus et al., 2012).

287 To mitigate random false positives resulting from antibodies unrelated to the
288 immunization with the SN-1 venom fraction, the peptide microarray was incubated
289 twice, starting with serum from the rabbit drawn before immunization, followed by
290 incubation of serum obtained after the last immunization. This double incubation setup
291 is described in more details

A secondary anti-rabbit IgG antibody was used to obtain signals from each of the two serum binding steps. The pairs of signals obtained for each peptide after the two incubation steps were correlated (Fig 2A), and the addition of immunized rabbit serum and anti-rabbit IgG antibodies to the microarray was found to increase unspecific signals with a factor of 1.5 (dashed line in Fig 2A). Every signal from the serum of the immunized rabbit was corrected by subtraction of the corresponding signal from the pre-immune serum multiplied by this "reincubation effect".

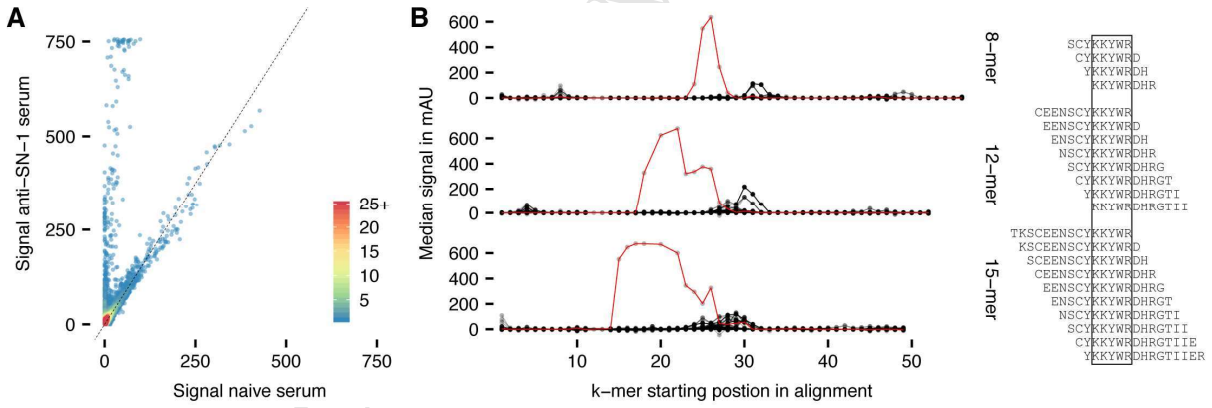

Figure 2. A) *Correlation of signals from all k-mer peptides obtained after incubation of microarray with pre-immune serum and with anti-SN-1 serum. The peptides of interest have low signals when the pre-immune serum is added, but high signals when anti-SN-1 serum is added, thereby revealing a binding event between peptides and antibodies resulting from the immunization*

procedure. Points are colored according to scale to indicate the number of peptides found to have identical signal intensities in both incubations. ***B)*** *Aligned binding profiles for 118 short-chain (type-1) α-neurotoxins. Signals of k-mer peptides were mapped to the sequences of the α-neurotoxins according to the position of the N-terminal residue of each k-mer in the multiple sequence alignment. SN-1 is highlighted as a red line. For each k-mer size, a single peak in the binding profile for SN-1 is found ending in alignment position number 27 indicating detection of one linear epitope element in SN-1. Peptides from SN-1 with scores above 100 are aligned in the right part of the figure, showing that the same five residues are present in all recognized peptides. The smaller peaks in black ending in alignment position number 32 for all three k-mer lengths reveal a separate and less recognized linear epitope element present in 12 related α-neurotoxins.*

The corrected signals were first mapped to the primary sequence of SN-1 and the 117 other short-chain (type 1) α-neurotoxins included in the study. The results are displayed as multiple sequence aligned superimposed binding profiles in Fig 2B, resembling the data representation in our previous studies of commercial antivenoms (Engmark et al., 2016, 2017). The binding profiles of each of the different peptide lengths (8, 12, and 15 amino acid residues) clearly demonstrate that SN-1 was the best

322 recognized short-chain α-neurotoxin. Also, the overlap between the recognized
323 peptides were almost identical for the three different peptide lengths demonstrating
324 the localization of a single linear epitope element, KKYWR, in SN-1 (Fig 2B).

325 The results also show identification of a linear epitope element in twelve short-
326 chain α-neurotoxins from cobra species. This linear epitope element was detected in a
327 subsequent site (8-mer binding profile in Fig 2B), closely resembling an unrecognized
328 sequence of SN-1 (Fig 3A). The central sequence (DHRG) in the recognized peptides is
329 present in most sequenced short-chain α-neurotoxins and in no other snake venom
330 protein according to BLAST search. The detection of this small amount of cross-
331 reactivity could indicate that one of the impurities in the venom fraction used for
332 rabbit immunization (Fig S1) may be another black mamba short-chain α-neurotoxin,
333 which could have induced antibodies recognizing similar cobra toxins. This cross-
334 recognition of cobra short-chain α-neurotoxin might explain why some of the cobra
335 venoms are recognized – although to a very low extent – in the ELISA experiment (Fig
336 1C). The best recognized cobra venom in the ELISA experiments is the venom of the
337 forest cobra, *N. melanoleuca,* which is known to have many short-chain α-neurotoxins

in its venom (Lauridsen et al., 2016) in opposition to for example the monocled cobra (*Naja kaouthia*), whose venom is dominated by long-chain α-neurotoxins (Laustsen et al., 2015a; Tan et al., 2015), which is almost not recognized (Fig 1C).

The shared residues between the highly-recognized overlapping peptides mapped to the SN-1 sequence suggest that the sequence KKYWR in alignment position 27 to 31 is recognized by the antibodies in the rabbit antiserum (Fig 2B). Only the tyrosine in position 29 is not conserved in the corresponding sequences of the cobra α-neurotoxins (Fig 3A), demonstrating that this amino acid is likely to be key for antibody recognition in this site of short-chain α-neurotoxins. As the tyrosine in this position is needed for antibody recognition in the site and as it is only reported in SN-1 from black mamba, the antiserum is highly selective for SN-1 in this linear epitope element.

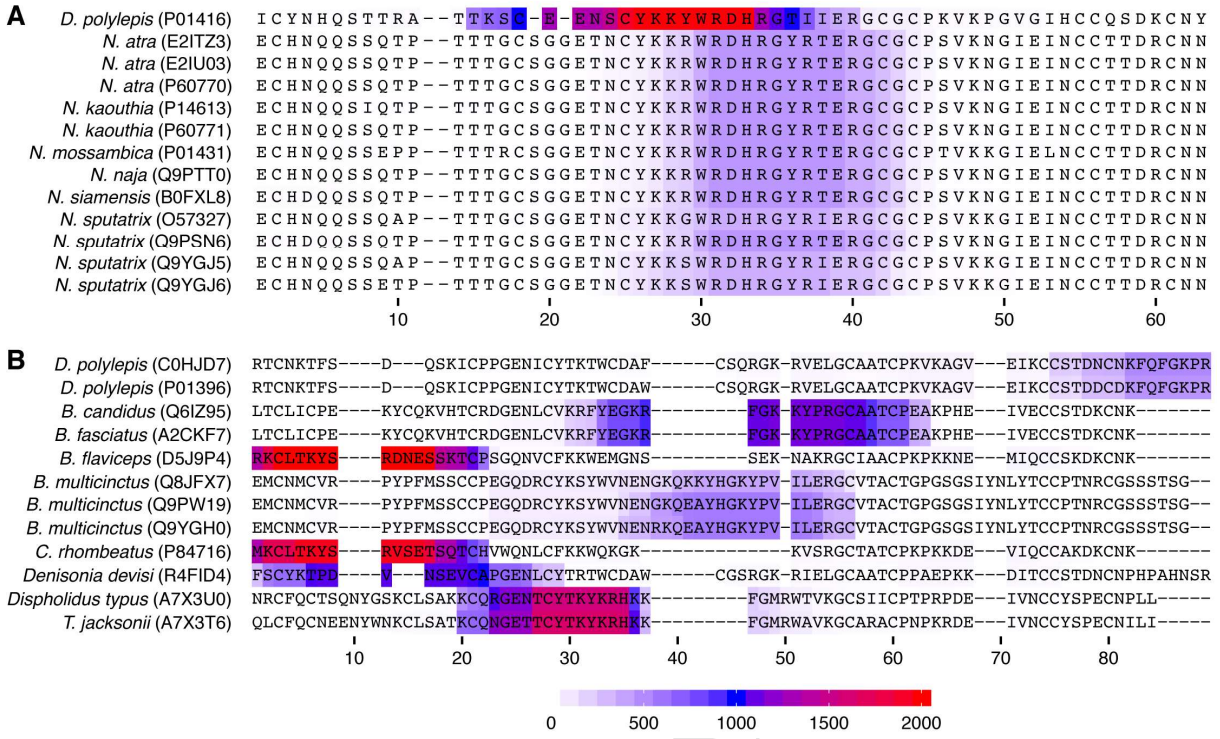

***Figure 3**. Linear epitope elements recognized by the anti-SN-1 serum. Mapping of signals to the protein sequences of the best recognized 3FTxs. Each residue is colored according to the sum of the best recognized 8-mer, 12-mer, and 15-mer containing the residue and thereby reflecting how well peptides containing the residue are recognized by the SN-1 antiserum. **A)** The top 13 (out of 118) best recognized short-chain α-neurotoxins including black mamba SN-1 (P01416). **B)** The top 12 (out of 634) best recognized 3FTxs not belonging to the group of short-chain α-neurotoxins. The two D. polylepis toxins on the top of the list are highly similar long-chain (type 2) α-neurotoxins, which could possibly have been present in trace amounts in fraction 4 used for immunization.*

Besides the investigation of peptides from 118 short-chain α-neurotoxins, 624 sequences from other 3FTxs and 10 Kunitz-type serine protease inhibitors (dendrotoxins) were included in the experimental setup. Out of this number, 12 3FTxs not belonging to the short-chain α-neurotoxin subfamily were found to be recognized by the rabbit antiserum, as defined by having a least two overlapping peptides with a background-corrected signal above 100 mAU (Fig 3B). No dendrotoxins were found to pass this threshold indicating no cross-reactivity to any of the investigated dendrotoxins. Two of the recognized 3FTxs are long-chain (type 2) α-neurotoxins originating from black mamba venom. These may have been recognized due to the presence of trace amounts of a long-chain α-neurotoxin in venom fraction 4 (Fig S1) used in the immunization procedure, causing a weak antibody response against these toxins. An alternative explanation, however, is that the two black mamba long-chain α-neurotoxins are recognized in a motif (KKYPR) very similar to the KKYWR motif from SN-1 discussed above (Fig 3). Furthermore, the observed cross-recognition of peptides derived from toxins that are not long neurotoxins or even from the black mamba (Fig 3B) can be explained by the presence of similar motifs to KKYWR: TKYSR and TKYKR.

375 This indicates that the anti-SN-1 serum may recognize a linear epitope element
376 described as K/T-K-Y-X-R, with X being an unspecified amino acid residue.

377 In our previous high-density microarray study of a polyvalent and black mamba-
378 specific antivenom from South African Vaccine Producers, the tyrosine in the KKYWR
379 motif was likewise found to be important for binding (Engmark et al., 2016). Moreover,
380 the four subsequent residues, DHRG, recognized in the cobra short-chain α-
381 neurotoxins by the rabbit antiserum (Fig 3A), were also found to be a linear epitope
382 elements in the study of the antivenom (Fig 4A). These two successive motifs are
383 localized to the loop 2 in the structure of SN-1 (Fig 4B), suggesting that loop 2 for this
384 group of 3FTxs is likely to be a preferred recognition site for antibodies in both horses
385 and this rabbit (Fig 4B). Whether this observation reflects a convergence between the
386 immune systems in rabbits and horses, or if it just reflects individual variation in the
387 immune response of a single animal cannot be determined from the current data and
388 is beyond the scope of this study. The finding does, however, highlight that polyclonal
389 antibody responses may generally be subject to variation on both an interspecies and

intraspecies level, making identification of broadly recognized linear epitope elements challenging.

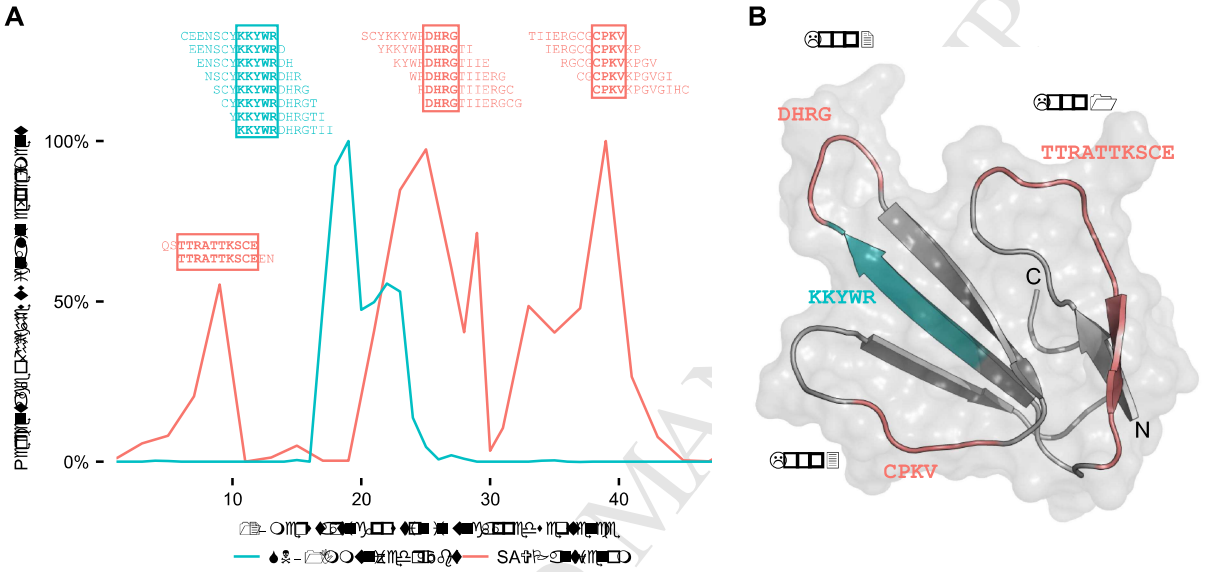

Figure 4*. Mapping of 12-mer results from this study and a previous study of a polyvalent antivenom to the primary sequence and structure of SN-1.* ***A)*** *To allow comparison of the peptide-signal data for the two experiments, the signals are normalized to a percentage of the highest signal in each binding profile. The 12-mer peptides recognized by the antivenom/rabbit antiserum are aligned, and shared amino acid residues highlighted in boxes. Notice that the overlap between neighbouring peptides are not identical in the two experiments.* ***B)*** *The overlapping amino acid residues identified in A) mapped to an experimental structure of SN-1, PDB accession number 1NTX (Labhardt et al., 1988). The recognized motifs in SN-1 is labeled and colored according to A).*

Conclusion

In this study, a rabbit was immunized over a period of 3.5 months with the highly toxic fraction 4 from the venom of the black mamba (*D. polylepis*), containing short neurotoxin 1 (SN-1). The antiserum was found to recognize one linear epitope element in SN-1, identified by a high-density peptide microarray approach using peptides of three different lengths (8-mers, 12-mers, and 15-mers). The antiserum was found to elicit very limited cross-reactivity to the majority of the 751 sequences of other 3FTxs and dendrotoxins studied in the setup. In general, other toxins of the same subfamily as SN-1 were not recognized in the same site as where the linear epitope element of SN-1 occurred, although only one amino acid residue distinguished the most recognized 8-mer peptides of SN-1 from 8-mers derived from homologous toxins. This indicates a remarkably high selectivity of the antibodies raised in the rabbit against the SN-1 toxin. This is in opposition to the polyvalent antivenoms previously investigated in peptide microarrays (De-Simone et al., 2013; Engmark et al., 2016, 2017). Although

417 short-chain 3FTxs are famous for being poorly immunogenic and the results may not
418 be generalized to other single toxin immunizations, we speculate that immunization
419 with multiple similar toxins may enhance cross-reactivity of polyclonal antibodies. This
420 may allow antibodies to become more tolerant to amino acid substitutions and
421 recognize a wider range of homologous toxins. To further explore this hypothesis,
422 further investigation using the high-density microarray approach are, however, needed.

423 This exploratory study demonstrates that cross-reactivity even to highly similar
424 toxins is not necessarily easily obtained when immunizing an animal with a single toxin.
425 The use of mixtures of whole venoms is likely to increase the extent of cross-
426 recognition. However, this may possibly also create an undesirable bias towards
427 proteins of less medical importance, as these that cannot easily be separated from the
428 medically relevant venom toxins. In turn, this can result in a lower concentration of
429 therapeutically active antibodies in a serum. An alternative strategy could, however, be
430 pursued. By selecting a mixture of isolated or recombinantly expressed neurotoxins and
431 using these for immunization, it may potentially be possible to obtain a therapeutically

enhanced antivenom with broad cross-reactivity against several elapid venom toxins, but with limited reactivity against medically irrelevant venom proteins.

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448 ## Conflicts of Interest

449 The authors declare no conflict of interest. The funding sponsors had no role in the
450 design of the study; in the collection, analyses, or interpretation of data; in the writing
451 of the manuscript, and in the decision to publish the results.

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Highlights

- Antiserum cross-reactivity may be limited when immunizing with a single neurotoxin
- Antibody-toxin binding may be lost upon substitution of one toxin epitope residue
- Linear epitope mapping results are consistent when using different peptide lengths

Ethical statement

Related to the manuscript entitled '*Exploration of the antibody response against a short-chain alpha-neurotoxin using high-density peptide microarray'.*

Before the study was initiated, the protocol of rabbit immunization was approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica